

Human Germinal Center CD4⁺CD57⁺ T Cells Act Differently On B Cells Than Do Classical T-Helper Cells

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We have isolated two subtypes of helper T cells from human tonsils: CD4⁺CD57⁺ cells, mostly located in the germinal center (GC), and CD4⁺CD57⁻ cells, distributed through the interfollicular areas but also present in the GC. In a functional study, we have compared the capacities of these T-cell subtypes to stimulate B cells in cocultures. In order to block T-cell proliferation while maintaining their activation level, we pretreated isolated T cells with mitomycin C prior to culture in the presence of B cells and added polyclonal activators such as PHA and Con A, combined or not with IL-2. Contrary to CD4⁺CD57⁻ cells, CD4⁺CD57⁺ cells did not markedly enhance B-cell proliferation. Even when sIgD⁺B cells typical of germinal center cells were tested, the CD4⁺CD57⁺ cells had no significant effect. This is in accordance with the location of these cells: They mainly occupy the light zones of the GC where few B cells divide. Even when added to preactivated, actively proliferating cells, CD4⁺CD57⁺ cells failed to modulate B-cell multiplication. On the supernatants of B-cell-T-cell cocultures, we examined by the ELISA technique the effect of T cells on Ig synthesis. Contrary to CD57⁻ T cells, whose effect was strong, CD57⁺ T cells weakly stimulated Ig synthesis. More IgM than IgG was generally found. Because CD57 antigen is a typical marker of natural killer cells, we tested the cytolytic activity of tonsillar CD4⁺CD57⁺ cells on K562 target cells. Unlike NK cells, neither CD4⁺CD57⁺ nor CD4⁺CD57⁻ cells exhibit any cytotoxicity. Thus, germinal center CD4⁺CD57⁺ cells are not cytolytic and do not strongly stimulate either B-cell proliferation or Ig secretion. CD4⁺CD57⁻ cells, however, enhance B-cell proliferation and differentiation, thus acting like the classical helper cells of the T-dependent areas.

KEYWORDS: Germinal center, T-helper cells, CD57⁺ T cells, cell proliferation.

INTRODUCTION

T lymphocytes purified from human tonsils are mainly CD4⁺ cells (Sugiyama et al., 1976, 1984, 1987; Plum et al., 1987), CD8⁺ T cells being proportionally less numerous in tonsils than in the peripheral blood (Sugiyama et al., 1987). The prevalence of CD4⁺ cells in tonsils suggests that tonsillar T lymphocytes are functionally oriented to helper activity (Sugiyama et al., 1987). Several immunohistochemical studies have revealed the existence of tonsillar T cells expressing the CD57 antigen characteristic of cells with NK activity (Okada et al., 1988). These cells are essentially located in the GC light zone; a few are also found in the interfollicular

T-dependent areas and the mantle zones (Bouzahzah et al., 1993). In a previous ultrastructural morphological study, we have shown that these cells are medium-sized and do not contain the large granules typical of blood NK cells (Bouzahzah et al., 1993). Phenotypically, these cells do not express Leu 8, CD16, or CD11b, so they clearly differ from classical NK cells (Si and Whiteside, 1983; Verlardi et al., 1985, 1986a). These CD4⁺CD57⁺ cells do not express CD25 (IL-2 receptor), CD71 (transferrin receptor), or HLA-DR, but do express CD69 (Bowen et al., 1991). Thus, they should be viewed as preactivated rather than fully activated cells. Their ability to produce cytokines is much debated (Velardi et al., 1986b; Bosseoir et al., 1989, 1991; Bowen et al., 1991; Butch 1993).

There is no available data as to the function of CD4⁺CD57⁺ cells. In this report, we compare the effects of CD4⁺CD57⁺ and CD4⁺CD57⁻ cells on

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B-cell proliferation and differentiation in Ig-secreting cells. Using several procedures (nylon wool columns, and selections by dynabeads and MACS), we have purified both populations of T-helper cells and cocultured them with B cells isolated from tonsils. We have compared the capacity of B cells to incorporate tritiated thymidine or to produce IgM or IgG in these two types of coculture. We pretreated the T-cell populations with mitomycin C to prevent their multiplication but added various activators to the cultures. In another series of experiments, we tested the cytotoxicity of CD4⁺CD57⁻ and CD4⁺CD57⁺ in the presence of K562 target cells.

RESULTS

Effects of CD57⁺ and CD57⁻ T Cells on B-cell Proliferation

To test the effects of CD57⁺ and CD57⁻ cells on B-cell proliferation, cell populations enriched in these cells were added to highly purified B cells. The purity of the T-cell populations used, as estimated by flow cytometry, exceeded 94% (CD57⁺ cells) or 96% (CD57⁻ cells). Before being cocultured, the T-cell populations were treated with mitomycin C to minimize their capacity to incorporate [³H]-thymidine. The cells were stimulated, however, with PHA (2 µg/ml) to keep them activated. As a control, B cells were cultured alone. Little or no proliferative activity was observed in B-cell populations cultured alone or in mitomycin-C-treated T cells. Coculturing with unsorted CD4⁺ T cells or with purified CD4⁺CD57⁻ cells markedly increased B-cell proliferation (Fig. 1). CD57⁺ cells had only a very slight enhancing effect on B-cell proliferation. Next, we reasoned that germinal center B cells might react differently than the total B-cell population. Therefore, after eliminating IgD⁺ cells from the total tonsillar B-cell population, we cocultured either the total B-cell population or the sorted IgD⁻ B cells with mitomycin-C-treated T cells in the presence of PHA (Fig. 2). The results obtained were similar to those of the previous experiments: the CD57⁺T cells exerted only a weak enhancing effect on B or IgD⁻ cell proliferation in comparison to the marked effect of CD57⁻ T cells.

The previous experiments showed that CD57⁺ T cells only slightly increase the proliferative activity of B cells. Considering that these CD57⁺ cells might

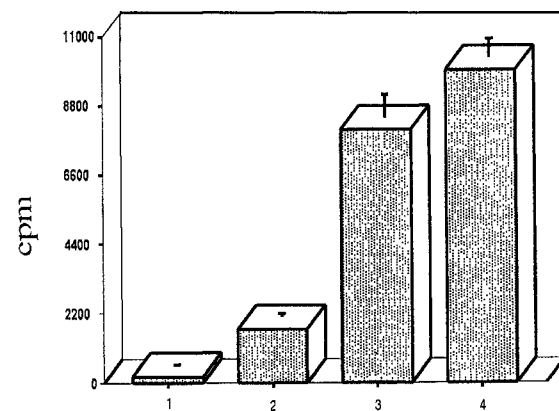


FIGURE 1. Proliferation of B cells in presence of CD57⁺ or CD57⁻ cells. B cells (10^5 cells per well) were incubated with the CD57⁺ or CD57⁻ cells (3×10^5 cells/well) and examined for proliferation after 48 hours of culture. The CD57⁺ and CD57⁻ cells were pretreated with mitomycin C (50 µg/ml for 45 min) and activated with PHA (2 µg/ml). Levels of tritiated thymidine uptake are indicated in CPM. The results are expressed as the mean of quadruplicate cultures and are representative of 4 experiments performed. In 1: B cells alone; 2: B cells and CD57⁺ cells; 3: B cells and CD57⁻ cells; 4: B cells and unsorted CD4⁺ cells.

not be sensitive to PHA, we performed a new series of experiments in which mitomycin-C-treated CD57⁺ or CD57⁻ cells were cocultured with B cells in the presence of PHA or ConA, combined or not with IL-2. B-cell proliferation was measured after 48 hours (Fig. 3). Addition of IL-2 to PHA only slightly improved the uptake of tritiated thymidine by B cells cocultured with the CD57⁺ cells. A much better response was measured when these cells were stimulated with ConA and IL-2. Similar results were obtained in cocultures of B and CD57⁻ T cells, but the level of proliferation was greater than in cultures composed of B and CD57⁺ cells. None of the activators used (ConA, PHA, IL-2) elicited any significant proliferative response in B cells cultured without T cells.

Effects of CD57⁺ and CD57⁻ Cells on the Proliferative Activity of Preactivated B Cells

The previous experiments were performed with freshly isolated B and T cells. To see whether the T-cell population might inhibit active B-cell proliferation, the B cells were preactivated for 24 hours with SAC or with anti-CD40 antibody combined with anti-Ig (respectively at 100 ng/ml and 1/600). The sorted T cells were treated with mitomycin C before coculturing with preactivated B cells. PHA was included throughout the culture period.

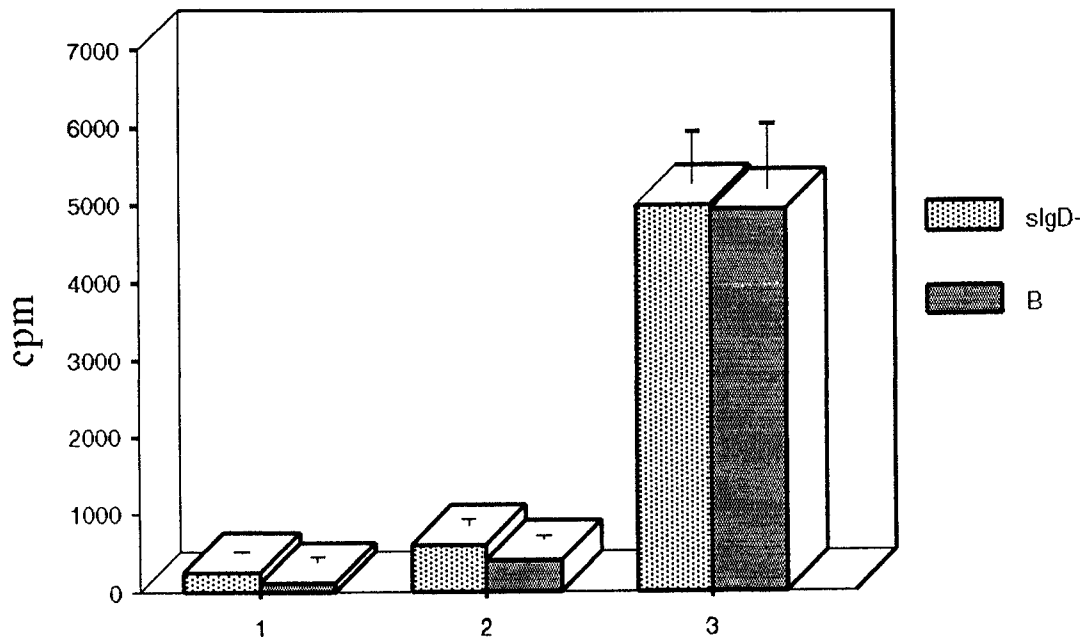


FIGURE 2. Proliferation of unsorted B cells or IgD⁻B cells in presence of CD57⁺ or CD57⁻ cells. B cells or sIgD⁻B cells (10^5 cell per well) were cocultured with mitomycin-C-treated CD57⁺ or CD57⁻ cells (3×10^5 g/well). sIgD⁻ cells were sorted using anti-IgD⁻ mAb and the MACS procedure. PHA was added whole during culture duration to maintain T-cell activation. B-cell proliferation was measured after 48 hours. The results are expressed as the mean of quadruplicate cultures. In 1: B cells and sIgD⁻ cells alone; 2: B cells and sIgD⁻ cells and CD57⁺; 3: B cells and sIgD⁻ cells and CD57⁻.

As shown in Fig. 4, the proliferation of B cells alone was markedly increased in the presence of all the different activators. Addition of CD57⁺ cells increased the proliferative response when the B cells were stimulated with SAC, but not when they were preactivated with anti-Ig and anti-CD40. Addition of CD57⁻ T cells was always followed by marked stimulation of the preactivated B cells.

Effects of CD57⁺ or CD57⁻ T Cells on Ig Secretion

Tonsillar B cells were cultured alone or in the presence of mitomycin treated CD57⁺ or CD57⁻ T cells. The ratio of T to B cells was 3/1. PHA or ConA, combined or not with IL-2, was added as a stimulator to the culture medium. The supernatants

TABLE 1
Effect of CD57⁺ or CD57⁻ Cells on Ig Production

Addition to Culture Medium	Secreted Ig (ng/ml)	
	IgM	IgG
B cells + PHA + IL2	600 ± 48	994 ± 180
B cells + ConA + IL2	600 ± 48	< 733
B cells alone	600 ± 48	760 ± 225
B cells + CD57 ⁺ cells + PHA	600 ± 48	922 ± 180
B cells + CD57 ⁺ cells + PHA + IL2	3,216 ± 211	1,899 ± 225
B cells + CD57 ⁺ cells + ConA	2,544 ± 192	594 ± 57,6
B cells + CD57 ⁺ cells + ConA + IL2	3,609 ± 240	877 ± 180
B cells + CD57 ⁻ cells + PHA	3,580 ± 259	3,271 ± 495
B cells + CD57 ⁻ cells + PHA + IL2	10,170 ± 1,350	5,566 ± 644
B cells + CD57 ⁻ cells + ConA	3,926 ± 384	< 733
B cells + CD57 ⁻ cells + ConA + IL2	11,475 ± 1,485	733 ± 180

Purified tonsillar B cells (1×10^5) were cocultured with mitomycin-C-treated CD57⁺ or CD57⁻ cells (3×10^5) in 96-well plates in a total volume of 200 μ l per well in presence of different combinations of activators (PHA; 2 μ g/ml; ConA: 10 μ g/ml; IL-2: 10 U/ml). After 10 days, the culture supernatants were harvested and analyzed for the presence of IgG and IgM by specific ELISA. The results are representative of 3 experiments.

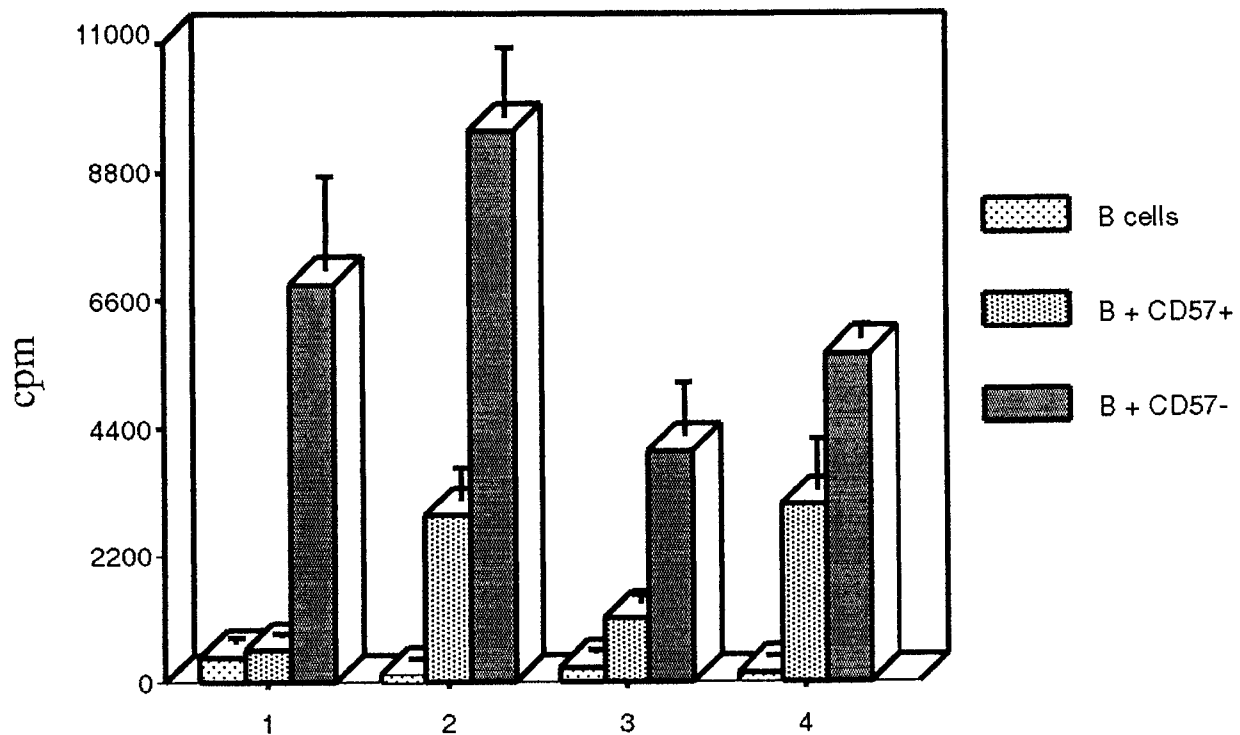


FIGURE 3. Proliferation of B cells in presence of CD57⁺ or CD57⁻ cells and different activators. B cells (10^5 cells/well) were cocultured with mitomycin-C-treated T cells (3×10^5 cells/well). Tritiated thymidin incorporation was measured after 48 hours. No or very low proliferative activity was observed in B cells cultured alone or in mitomycin-C-treated T-cell populations. T cells (CD57⁺ or CD57⁻) were activated with PHA (2 μ g/ml) or Con A (10 μ g/ml) combined or not to IL-2 (10 U/ml): 1: PHA; 2: PHA and IL-2; 3: ConA; 4: ConA and IL-2. These results are representative of 3 experiments performed and are expressed as the mean of quadruplicate cultures.

were collected after 10 days of culture and their IgM and IgG contents analyzed by ELISA. The results are presented in Table 1, B cells cultured alone did not produce large amounts of Ig. Addition of T cells generally increased Ig synthesis, CD57⁻ T cells being much more effective than CD57⁺ T cells. IgM were secreted in larger quantities than IgG, especially when IL-2 was present in the medium. ConA was a better inducer of IgM production than PHA, but less effective than PHA at increasing IgG secretion. Thus, IgM secretion was highest when B cells were cocultured with CD57⁻ T cells stimulated by ConA and IL-2, whereas IgG secretion was highest in B cells cocultured with CD57⁻ cells stimulated with PHA and IL-2.

Cytolytic Activity of CD57⁺ and CD57⁻ T Cells

Neither freshly isolated nor preactivated CD57⁺ or CD57⁻ cells exerted any cytolytic activity against

K562 target cells. Comparatively high cytolytic activity was measured in the positive controls: for example, 60% ⁵¹Cr release at 20:1 and 50% at 10:1 (effector: target ratio).

DISCUSSION

Helper activity toward B lymphocytes can efficiently be induced *in vitro* by stimulation of human T cells with various mitogens (Hirohata et al., 1988; Patel et al., 1993; Dembech et al., 1992). In this report, we examine the effect of CD4⁺CD57⁺ and CD4⁺CD57⁻ tonsillar T-cell populations on the proliferation and terminal differentiation of B cells isolated from tonsils.

The CD4⁺CD57⁻ cells are representative of the T-cell populations of the interfollicular areas and GC, whereas the CD4⁺CD57⁺ are essentially GC T cells. Although the CD57 Ag is also expressed by NK cells, the tonsillar CD57⁺ cells are morpholog-

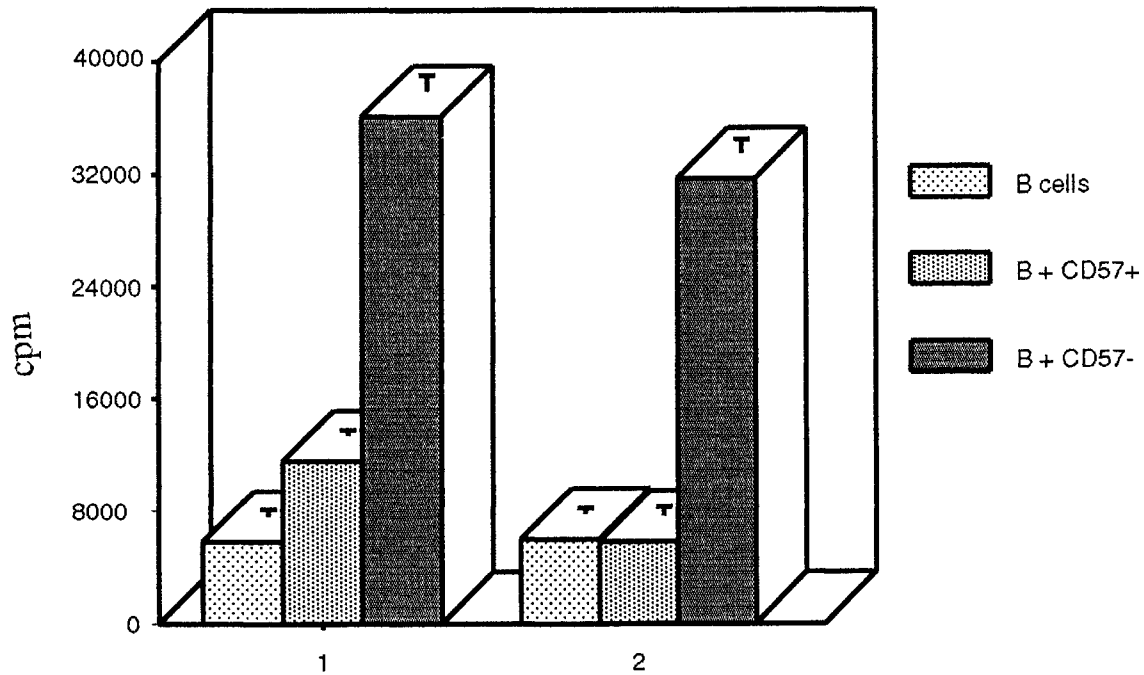


FIGURE 4. Effect of CD57⁺ or CD57⁻ cells on the proliferation of preactivated B cell. B cells (10^5 cells) were preactivated with: 1:SAC (at a final dilution of 1/10,000) or 2: anti-Ig combined to anti-CD40 (respectively at 1/600 and 100 ng/ml). After 24 hours of culture, mitomycin-C-treated CD57⁻ or CD57⁺ cells (3×10^5 cells/well) were added in presence of PHA (2 μ g/ml). Thymidine incorporation by B cells was measured after 48 hours. Results are expressed as the mean of quadruplicate cultures and are representative of 5 experiments.

ically and functionally different. We have, in fact, evaluated the cytolytic activity of these cells by the ^{51}Cr release method, using human K562 cell as targets. Unlike classical NK cells, tonsillar CD4⁺CD57⁺ manifest no NK activity, even when preactivated with PHA for 24 hours. Our results agree with those of Velardi et al. (1986a, 1986b). Thus, expression of this NK-related antigen does not reflect a cell's cytolytic potential, but characterizes a subset of CD4⁺ T-helper cells in germinal centers.

When CD57⁻ or CD57⁺ cells were cocultured with B cells, B-cell proliferation was slightly increased by the CD57⁺ cells but enhanced thirty-fold in the presence of CD57⁻ cells or unsorted CD4⁺ cells. This finding is in line with results reported by Velardi et al. (1986b) showing that CD57⁺ cells do not produce B-cell growth factors. Several studies have indicated that a subpopulation of germinal center B cells can proliferate in response to low concentrations of IL-2 (Forman and Pure, 1991). In our experiments, CD57⁺ T cells did favor B-cell proliferation when IL-2 was added to the medium. IL-2 by itself was not sufficient to stimulate B-cell proliferation, because B cells cultured alone in the

presence of IL-2, combined or not with PHA or ConA, failed to proliferate. IL-2 appears to act on T cells, which then stimulate B cells. This activation requires also direct T-cell-B-cell interaction (Clark and Lane, 1991; Tohma and Lipsky, 1991; Liu et al., 1992; MacLennan et al., 1992; Moller, 1992) because physical separation of the two cell types with a semipermeable membrane precludes B-cell activation. Cytokines, particularly IL-2 (Porwit-Ksiazeck et al., 1983) and IL-4 (Fliedner et al., 1964; Defrance et al., 1988), appear to play an important role. The capacity of CD57⁺ cells to produce IL-2, IL-4, and other cytokines is much debated: Bosseloir et al., (1989, 1991) using *in situ* hybridization failed to detect any IL-6 or IL-4-secreting cells in tonsil germinal centers, whereas Butch et al. (1993) say they have detected IL-4 mRNA in GC T cells. Currently, we are checking the presence of cytokines in supernatants of CD57⁺ cell cultures and analyzing mRNA contents of freshly prepared CD57⁺ T cells.

At this level of our work, we can conclude that CD57⁺ T cells, contrary to CD57⁻ T cells, whose effect is strong, exert a very weak effect on B-cell

proliferation, even on IgD⁻ B cells arising in part in the germinal centers. This conclusion is in agreement with immunohistological studies showing that CD57⁺ T cells are predominantly located in the germinal center light zone. One should remember that B-cell proliferation occurs mainly in the dark zone (O'Garra et al., 1988; Freedman et al., 1992), where few CD57⁺ T cells are found. It has been suggested that in the light zone, differentiation of B cells into memory cells or plasma cell precursors is accompanied by decreased B-cell proliferation (Noëlle and Snow, 1992). Because CD4⁺CD57⁺ cells are numerous in the light zone, we reasoned that they might play a role in curbing the proliferative activity of dividing B cells coming from the dark zone. We tested this by coculturing the two T-cell populations with B cells preactivated for 24 hours with SAC or anti-CD40 combined with anti-Ig. The CD57⁻ T cells were found to markedly enhance B-cell proliferation whatever the stimulator used; CD57⁺ cells, on the contrary, only slightly increased B-cell proliferation in the presence of SAC and had no effect on B cells preactivated with anti-Ig and anti-CD40.

In another series of experiments, we have shown CD57⁻ T cells to be much more effective than CD57⁺ cells in inducing B cells to secrete IgM or IgG. This action of T cells on Ig secretion was largely dependent upon the mode of stimulation applied. ConA, for instance, was a better inducer of IgM synthesis than PHA, whereas Ig production was highest in the presence of IL-2. B cells cultured alone exhibited only minimal Ig secretion, even in the presence of exogenous IL-2 combined or not with ConA or PHA. In fact, Dembech et al. (1992) report having failed to induce B cells to secrete significant amounts of Ig by adding various recombinant cytokines, such as IL-2, IL-4, IL-6, IFN α , all involved in the proliferation and differentiation of B cells (O'Garra et al., 1988). Thus, optimal differentiation of human B cells appears to require not only cytokines, but also close contact with T cells (Reidel et al., 1988; Whalen et al., 1988; Noëlle and Snow, 1992). One of the molecules involved in T-cell-B-cell contact is CD40. Expression of CD40 ligand apparently occurs in activated T cells, but expression of this molecule by GC T cells (CD4⁺CD57⁺) remains controversial (Spriggs et al., 1993; Van den Eertwegh et al., 1993).

We have thus defined two populations of tonsillar CD4⁺ cells that differently affect B-cell proliferation and Ig production. The functional differences be-

tween the CD4⁺CD57⁺ and CD4⁺CD57⁻ populations may be due to the ability of these cells to respond to the mitogens used. We are currently continuing to test the proliferative capacity and the expression of activation markers by these cells in response to different mitogens and accessory cells.

Further studies must be conducted to investigate the function of CD57⁺ T cells, notably in cocultures where follicular dendritic cells are present as well as centrocytes. Indeed, CD57⁺ cells in the light zone are adjacent to centrocytes that appear as intermediates between activated centroblasts (dark zone) and quiescent B memory cells (recirculating lymphocytes). We hypothesize that CD57⁺ T cells may contribute to this maturation of centrocytes into B memory lymphocytes.

MATERIAL AND METHODS

Reagents

Collagenase, dispase, and deoxyribonuclease were obtained from Boehringer Mannheim and used at 0.005%. Magnetic beads and dynabeads coated with anti-mouse IgG were purchased respectively from Miltenyi Biotec (Germany) and Dynal (Norway). Mitomycin C was purchased from SIGMA and used at 50 μ g/ml. Phytohemagglutinin (PHA, 2 μ g/ml), concanavalin A (ConA, 10 μ g/ml), and purified recombinant IL-2 (10 U/ml) were purchased from Boehringer Mannheim (Germany). Formalinized particles of *Staphylococcus aureus* strain Cowan I (SAC) were purchased as pansorbin from Calbiochem-Behring Corporation (La Jolla, CA) and used at the final dilution of 1/10,000. Nylon wool was kindly provided by Dr. Thierry Defrance (Institut Pasteur de Lyon, France).

Monoclonal Antibodies

PE-conjugated and unconjugated anti-CD19 and anti-CD8, PE-conjugated anti-CD2 and anti-CD3 mAbs were purchased from DAKO. FITC-conjugated streptavidin was purchased from Boehringer Mannheim and used at the final dilution of 1/40. Biotin-conjugated anti-CD57 mAb was obtained from Becton Dickinson. Culture supernatants of OKT3 (anti-CD3 mAb) and OKT 11 (anti-CD2 mAb) hybridoma cell lines and the anti-CD40 mAb were kindly provided by Dr. E. A. Clark (University of Washington, Scolt, WA). Rabbit anti-human

immunoglobulin coupled to polyacrylamide beads was purchased from Biorad Laboratories (Richmond, CA) and used at the final dilution of 1/600. Anti-IgD m Ab (Amersham) was used at the final dilution of 1/300.

Isolation Procedure of Helper T Cells

Tonsils from 4- to 1-year-old children were surgically dissected and transported to the laboratory at 4°C in a physiological solution containing 0.4% bovine serum albumin (BSA). The tonsils were then cut into fragments and treated first with collagenase alone, then with a mixture of collagenase, dispase, and deoxyribonuclease.

T cells were separated from other cells by running the lymphocyte suspensions through nylon wool columns prepared by packing 50-ml syringes with nylon wool. Before the cells were added (10^9 cells per column), the columns were washed at 37°C with RPMI 1640 supplemented with 10% FCS. After the cells were added, the columns were eluted with RPMI-10% FCS until the cells had run two-thirds of the way through them. After incubation for 1 hour at 37°C, the columns were slowly rinsed with the eluent (RPMI-10% FCS at 37°C) and the nonadherent T cells recovered. The recovered T cells were labeled with anti-CD8 and anti-CD19 mAbs and subsequently incubated with anti-mouse IgG-coated magnetic beads. The B cells and CD8⁺ cells were removed by applying a magnetic field for 10 min. The purity of the CD4⁺ T-cell population exceeded 98%, as estimated by flow cytometry. An anti-CD57 mAb and a magnetic cell sorter (Becton Dickinson) were used to sort the CD4⁺ T cells into CD4⁺CD57⁺ and CD4⁺CD57⁻ cells. Briefly, the CD4⁺ T cells were incubated with biotin-conjugated anti-CD57 mAb for 30 min, rinsed, and placed in contact with FITC-conjugated streptavidin for 10 min. Then they were incubated with biotinylated magnetic beads before transfer to the separation column installed in the magnetic field of the MACS apparatus. The CD57⁺ cells were recovered by rinsing the column with a physiological solution at 4°C outside the magnetic field. As estimated by flow cytometry, the purity of the CD57⁺ and the CD57⁻ cells obtained by this procedure exceeded 94 and 96%. These T cells were treated with mitomycin C at 37°C for 45 min, rinsed thrice, then cocultured with B cells in the presence of PHA or ConA, combined or not with IL-2.

B-Cell Separation

After enzymatic digestion, tonsillar mononuclear cells were incubated with 2-aminoethylisothiouonium-bromide-treated SRBC. Nonrosetting cells were labeled with anti-T-cell mAbs (OKT3, OKT11) and dynabeads to remove contaminant T cells. As estimated by flow cytometry with PE-conjugated mAbs (anti-CD19, anti-CD3, anti-CD2), the purity of the B-cell populations thus obtained exceeded 95%. Anti-IgD mAb and MACS were used to isolate sIgD-B cells; the purity of the sIgD⁻ B cells, as estimated by flow cytometry, was 80%. All purification steps (rosetting, MACS) were performed at 4°C.

Cultures

Cultures were grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

1×10^5 B cell swere cocultured with 3×10^5 T cells per well in 96-well microtiter plates in a final volume of 150 µl (proliferation tests) or 200 µl (differentiation tests). The T cells were treated before hand with mitomycin C (50 µg/ml) at 37°C for 45 min. Different polyclonal T-cell activators (PHA, ConA, IL-2) were added to the cultures. To test the capacity of T cells to suppress B-cell proliferation, we preincubated the B cells with SAC or anti-CD40 combined with anti-Ig for 24 hours prior to addition of T cells.

The cultures were incubated at 37°C in a 5% CO₂ enriched atmosphere for 48 hours. A B-cell proliferation was tested by pulsing the cells with [³H]thymidine (1 µCi per well) during an additional 16-hour incubation. Results were expressed as means of triplicate or quadruplicate cultures. To measure Ig production, we maintained the cultures for 10 days and analyzed the supernatants by the ELISA technique as described by Defrance et al. (1988).

Cytolytic Test

The cytolytic activity of CD57⁺ and CD57⁻ T cells was tested by measuring ⁵¹Cr release from human K562 cells as described by Parker and Martz (1980). Cytolytic activity was assayed on freshly isolated CD57⁺ and CD57⁻ cells or on ones pre-activated for 24 hours with PHA.

Activated peripheral blood lymphocytes (PBL) were used as positive controls.

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